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Development of novel flexible sugar ester vesicles as carrier systems for the antioxidant enzyme catalase for wound healing applications

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ABSTRACT

The antioxidant enzyme catalase (CAT) was encapsulated in biocompatible flexible non-ionic sugar esters (SEs) nano-vesicles for potential topical administration. The effects of the SE hydrophilic lipophilic balance (HLB) value and the carbon chain length of the fatty acid ester of different SEs on the encapsulation efficiency (EE) were studied. Morphology of the vesicles was not altered upon CAT encapsulation using freeze fracture electron microscopy. The extrusion measurements indicated that there was an increase in the vesicle's flexibility index upon the inclusion of phospholipids. The mean diameter of the CAT-EV (ester vesicle; HSC and HSC–PL) was 222–275 nm using laser diffraction measurements. The catalytic efficiency (V_{max}/K_m) of CAT was improved after encapsulation by a factor of 1.7. Both free CAT and CAT-EV showed maximum catalytic activity at pH 7.0, and CAT-EV was more stable than free CAT at acidic pH, which is advantageous for successful topical delivery. Encapsulation of CAT in SE vesicles protected it against trypsin treatment. Encapsulated CAT retained more than 60% residual activity after 12 successive decomposition cycles of H_2O_2. CAT-EV activity was significantly preserved compared to that of free CAT at 4 °C for 180 days. The *in vivo* study showed a significant effect of the prepared CAT nano-vesicles on wound healing.

1. Introduction

Catalase (CAT, H_2O_2 oxidoreductase, EC 1.11.1.6) is a complex homotetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa each. According to the nature of the catalytic group, catalases may be divided into heme-CAT or manganese-CAT [1]. CAT represents the most efficient and highly structured antioxidant system available in both plants and animals to control the hydrogen peroxide (H_2O_2) concentration. CAT is present mainly in the peroxisomes of mammalian cells where it efficiently catalyzes the decomposition of hydrogen peroxide to water and molecular oxygen with no free radical production. CAT has one of the highest turnover rates of all enzymes: one molecule of CAT can convert ~6 million molecules of hydrogen peroxide to water and oxygen/min [2].

A decreased level of CAT has been related to the significantly decreased capacity of a variety of tumors for detoxifying hydrogen peroxide [3]. Chapple [4] reported severe periodontal destruction in the condition of acatalasia, which consists of an inherited CAT deficiency in leukocytes and erythrocytes. In addition, CAT with hydrogen peroxide is also used as an antiseptic against anaerobes. Additionally, CAT is used for the treatment of hyperoxaluria [5]. CAT has been revealed as the most important factor in intrinsic aging and photoaging in human skin *in vivo* [6]. It has also been reported to be an important factor in inflammation [7] and the prevention of apoptosis [8]. Singh et al. [9] reported that *Plagiochasma appendiculatum* extract has potent wound-healing capacity as evidenced from the wound contraction and increased tensile strength. The results indicated that *P. appendiculatum* extract possesses potent antioxidant activity by inhibiting lipid peroxidation and increasing

Abbreviations: HSC, sucrose stearate sugar ester vesicles; CAT-EV, catalase encapsulating vesicles; S-370, S-970, S-1670, sucrose stearate esters; L-1695, sucrose laurate esters; M-1695, sucrose myristate esters; P-1670, sucrose palmitate esters; HLB, hydrophilic lipophilic balance; EE, Encapsulation efficiency; CAT, catalase; SE, sugar ester; Chol, cholesterol; SE-V, empty sugar ester vesicles.

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the superoxide dismutase (SOD) and catalase activity. Topical application of compounds with free-radical-scavenging properties in patients has shown significant improvement in wound healing and protection of tissues from oxidative damage [10].

The stabilization of multimeric enzymes is a particularly complex problem [11]. While in most monomeric enzymes the first step of their inactivation involves changes in their tertiary structure [12], the first step in the inactivation of multimeric enzymes is often the dissociation of the enzyme subunits or the loss of their correct assembled structure [13]. Several methods have been proposed to overcome these limitations. One of the most successful methods is enzyme immobilization [14]. Immobilization is achieved by fixing enzymes to -or within- various supports to obtain heterogeneous immobilized enzyme systems. Liposomes (lipid vesicles or phospholipid vesicles) and niosomes (hydrated mixture of non-ionic surfactants such as alkyl ethers, alkyl esters or alkyl amides and cholesterol) are able to carry hydrophilic drugs by encapsulation in the water phase or hydrophobic drugs by intercalation into hydrophobic domains [15]. Niosomes provide for sustained release and enhance the penetration of the trapped substances through the skin [16].

Sugar esters (SEs) are non-ionic surfactants prepared from natural and renewable resources with a sugar substituent, sucrose as a polar head group and fatty acids as apolar groups. SEs are biodegradable and biocompatible with low toxicity [17]. Sucrose has eight free hydroxyl groups, which allow researchers to devise many structural combinations suited for modulating surfactant properties from monoesters to octaesters with different fatty acids [18]. SEs have found numerous applications in many fields, such as cosmetics [19] and the food industry [20].

The aim of this study is to develop a new delivery system that allows for epicutaneous administration of the antioxidant enzyme catalase. Through the combination of the advantages of sugar esters and those of flexible vesicles, a new delivery system for CAT was developed. The second aim was to assess the wound-healing efficacy of the entrapment of CAT.

2. Materials and methods

2.1. Materials

Bovine liver catalase (CAT) (40,000–60,000 units/mg protein), hydrogen peroxide, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ryoto® SEs were provided by Mitsubishi Chemical Co. (Japan). Soybean phospholipid (PL) Lipoid S100-3 was a gift from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Chol) was purchased from Oxford Co. (England). All other chemicals were of analytical grade.

2.2. Preparation of CAT-encapsulating flexible sugar ester vesicles (CAT-EV)

SE-based vesicles were prepared by the thin–film hydration method [12]. SE, Chol, and PL were solubilized in chloroform/diethyl ether solvent mixture (1:1, v/v) in a dry 100 ml round-bottom flask. The solvent mixture was evaporated under reduced pressure at 60 °C using a rotary evaporator. The resulting film (20 mg/ml SE) was further dried overnight in a desiccator at room temperature (25 ± 2 °C). The obtained dried film was hydrated with 50 mM potassium phosphate buffer, pH 7.0 containing CAT (40 mg/ml, 180,000 U/ml) using mechanical agitation overnight at room temperature (25 ± 2 °C). The vesicular dispersion obtained was a mixture of free CAT and catalase encapsulating vesicles (CAT-EV), which consequently required further separation. The free CAT was separated from CAT-EV dispersion by repeated centrifugation at 45,000 rpm and 4 °C, for three consecutive runs 1 h each. Three types of CAT-EVs (HSC, HSC–P and HSC–PL) were prepared according to the different compositions used (Table 1). Removal of the free CAT was confirmed by testing CAT activity in the supernatant. After the final centrifugation run, the vesicles were resuspended in 50 mM potassium phosphate buffer, pH 7.0 and the catalytic activity was determined. Empty vesicles (SE-V) were prepared as described above but in the absence of CAT enzyme.

2.3. Determination of CAT activity and protein

CAT activity was assayed using a simple and quantitative spectrophotometric assay [21] using H_2O_2 as the substrate. The enzyme catalytic activity in CAT-EVs was determined after disruption of the vesicles with 0.5% (w/v) Triton X-100. The influence of 0.5% Triton X-100 on CAT activity was tested by assaying the free CAT activity in the presence of 0.5% Triton X-100 solution. Any effect that might be caused by Triton X-100 on the UV absorbance during the activity assay measurement was also compensated for preparing a control sample. The CAT-EV reaction was initiated by adding aliquots of prepared vesicular dispersion to a 20 mM H_2O_2 solution prepared in 50 mM potassium phosphate buffer, pH 7.0, to obtain a final CAT concentration of 0.2–0.4 μg/ml in the assay reaction mixture. Then, the time-course decomposition of the H_2O_2 solution into molecular oxygen and water catalyzed by CAT enzyme at 25 °C was followed at 240 nm using a spectrophotometer (JASCO V-530, Japan). The calculations were performed based on the H_2O_2 absorbance at 240 nm with the molar extinction coefficient of 43.6 M^{-1} cm^{-1}. For the free CAT activity measurements, the appropriate amount of the freshly diluted CAT samples was used as described above with a CAT concentration of 0.2–0.4 μg protein/ml in the assay reaction mixture. One unit of catalase is defined as the amount of enzyme that decomposes 1.0 μmol of H_2O_2 into molecular oxygen and water per min under standard assay conditions. The protein concentration was determined by the method of Bradford [22], using bovine serum albumin as a standard.

Table 1
Entrapment efficiency (EE) of CAT in sugar ester vesicles. Vesicles were prepared by the thin-film method using SE of different HLB values and different fatty acid esters at a fixed SE concentration of 20 mg/ml using S-PL.

SE	HLB	Weight ratio of SE-V components		
		SE:Chol:PL 10:1:10 HSC–P CAT (EE %)	SE:Chol:PL 4:4:1 HSC–PL	SE:Chol:PL 1:1:0 HSC
S-370	3	5.7 ± 0.53	16 ± 0.57	21.5 ± 0.6
S-970	9	19.4 ± 0.43	29.7 ± 0.54	35.5 ± 0.51
L-1695	16	20.7 ± 0.45	27.5 ± 0.46	30.6 ± 0. 54
M-1695	16	26.6 ± 0.34	33.6 ± 0.37	39.2 ± 0.31
P-1670	16	40.5 ± 0.31	50.2 ± 0.36	63.6 ± 0.45
S-1670	16	55 ± 0.21	70.8 ± 0.32	82 ± 0.45

Values are expressed as the mean ± SD (n = 3).

2.4. Determination of encapsulation efficiency (EE)

The encapsulation efficiency percent (EE%) was calculated according to the following equation:

$$EE\% = \frac{EC}{TC} \times 100$$

where EE% is the encapsulation efficiency percent, EC is the calculated encapsulated CAT concentration and TC is the total CAT concentration used.

2.5. Determination of SE-V flexibility

The flexibility of the prepared empty SE-V or CAT-EV was evaluated using the extrusion method, where the vesicles were transported across a semi-permeable barrier. The pore diameter was 3-times smaller than the average vesicle diameter. Empty SE-V or CAT-EV was extruded through polycarbonate membrane filters of decreasing pore diameters (400–100 nm; Avestin Inc., Canada). The size measurements were made before and after extrusion, and the vesicle dispersions were extruded for 21 consecutive passes. The extruded volume was measured using a syringe and the volumes were compared before and after extrusion. The final vesicle size and polydispersity index (PI) of the vesicle dispersion were determined by the dynamic light scattering (DLS) technique.

2.6. Measurement of vesicle size

Laser diffraction (LD) measurements were performed using the Mastersizer Hydro 2000S (Malvern Instruments, Malvern, UK). The calculations were based on Mie theory. The refractive index was set at 1.45 for the sample and 1.33 for the dispersion medium (water). The dimensional information gained by LD represents the mean of five successive measurements of 120 s. The LD measurements were expressed as the median volume diameter.

2.7. Freeze-fracture electron microscopy

Aliquots (approximately 10 μl) of freshly prepared vesicle dispersions were freeze-fixed using a propane jet-freeze device (JFD 030; BAL-TEC, Balzers, Liechtenstein). Thereafter, the samples were freeze-fractured at −150 °C without etching with a freeze-fracture/freeze-etching system (BAF 060; BAL-TEC, Balzers, Liechtenstein). The surfaces were shadowed with platinum to produce good topographic contrast (2 nm layer, shadowing angle 45°) and subsequently with carbon to stabilize the ultra-thin metal film (20 nm layers, shadowing angle 90°). The replicas were

floated in 4% sodium chloride for 30 min, rinsed in distilled water for 10 min, washed in 30% acetone for 30 min and rinsed again in distilled water for 10 min. Thereafter, the replicas were mounted on copper grids, coated with formvar film and observed with a transmission electron microscope (EM 900, Carl Zeiss SMT, Oberkochen) operating at 80 kV. Pictures were taken with a Variospeed SSCCD SM-1k-120 camera (TRS, Moorenweis, Germany).

2.8. Characterization of free CAT and CAT-EV

2.8.1. Intrinsic enzyme kinetics

Activity assays using H_2O_2 were carried out for the determination of kinetic parameters (Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max})) for the free CAT or CAT-EV. Measurements were performed using different concentrations of H_2O_2 ranging from 3 to 20 mM. The decomposition of each concentration of H_2O_2 was initiated at a fixed overall CAT concentration of 0.2 μg/ml. The CAT activity assay was carried out under standard assay conditions.

2.8.2. Thermal stability

The free CAT or CAT-EV was incubated for 15 min at various temperatures ranging from 20 to 90 °C. The tested samples were brought back to room temperature prior to substrate (H_2O_2) addition. The residual catalytic was determined under standard assay conditions as described above. The % residual catalytic activity was calculated as the proportion of the activity remaining with regard to the activity at 25 °C, which was taken as 100%.

2.8.3. pH optimum

The pH profiles for free CAT and CAT-EV were performed using the following buffers: 50 mM sodium acetate buffer for pH values 4.0 and 5.0 and 50 mM potassium phosphate buffer for pH values from 6.0 to 9.0 at 25 °C. The catalytic reactions were started by the addition of the proper dilution of free CAT or CAT-EV samples to the substrate solution.

2.8.4. Operational stability

The evaluation of the stability of the free CAT or CAT-EV activity against repeated decomposition of H_2O_2 was investigated according to the method described by Yoshimoto et al. [23]. The reaction mixture contained 20 mM H_2O_2, 50 mM potassium phosphate buffer, pH 7.0, and a fixed CAT concentration (0.2 μg/ml). Afterwards, the time course of H_2O_2 decomposition was followed until 0.2 OD decrease was recorded. Immediately aliquots of a freshly prepared 0.15 M H_2O_2 solution were added to yield 20 mM H_2O_2 in the reaction mixture to reproduce the initial reaction conditions. Successive H_2O_2 catalyzed decompositions were carried out. In addition, the effect of CAT concentration on the reusability of CAT-EVs was also investigated using the same procedure with different enzyme concentrations (0.02, 0.2 and 50 μg/ml).

2.8.5. Stability against proteolysis

Free CAT or CAT-EV at the same final concentrations was pre-incubated with different concentrations of trypsin (0–4 mg/ml) for 2 h at 37 °C in 50 mM potassium phosphate buffer, pH 7.0. After the incubation time, the catalytic reaction was started by the addition of H_2O_2 to the reaction mixture. H_2O_2 decomposition was followed spectrophotometrically under standard assay conditions.

2.9. Storage stability

The prepared CAT-EV and free CAT solutions (4 mg/ml) were stored at 5 °C in 50 mM potassium phosphate buffer, pH 7.0, in tightly closed Eppendorf tubes. Enzyme activity measurements were made periodically under standard assay conditions. The % residual activity was calculated as the proportion of the activity remaining at the end of the storage period compared to the initial activity at time zero, which was defined as 100%.

2.10. In vivo study: model of drug efficiency in wound healing

2.10.1. Experimental set-up

2.10.1.1. Animals. Clinical experiments involving animals were conducted according to the international guidelines and in accordance with the ethical committee guidelines at the German University in Cairo (GUC). Three groups of male Wistar rats (150–250 g), consisting of six animals each, were kept for 7 days of acclimatization. One day prior to each topical enzyme application, the hair on the selected dorsal area on the rat's skin was epilated.

2.10.1.2. Trauma Induction. The experimental design was performed according to the method described by Vorauer-Uhl et al. [24]. The animals were placed in a supine position inside a plastic box with an opening at the bottom. The animal was on its back, and its legs were tied out in full extension in a uniform and easily reproducible position. The trauma was performed by exposing of the epilated back skin of the anaesthetized animals (chloroform/ether) to hot water. A uniform deep second-degree burn on the back skin was produced with water at 95 ± 2 °C during a 30 s exposure. To clearly identify the original site of burn induction, the outline of the induced burn was marked accurately on the skin using a permanent marker.

2.10.1.3. Treatment. Treatment with CAT-EVs was performed immediately after trauma and once daily for 4 days (treated group). One group of animals was left untreated (naive group), and one was treated using empty SE-V (control group). The average CAT content in the applied CAT-EV was 4 mg/ml.

2.10.2. Experimental evaluation

The size of the lesions and wound contracture was evaluated at 24, 48, 72 and 96 h post trauma.

2.10.2.1. Size of lesions. The size of the lesions was determined as described by Vorauer-Uhl et al. [24], using a planimetric method that projects the lesions shapes onto a transparent pattern foil.

2.10.2.2. Wound contracture. After an initial lag period of 1 day, the rate of wound healing was followed visually by subjective evaluation using two inspectors. The inspectors were not notified about treated group to avoid bias in the evaluation.

2.11. Data analysis and statistics

Statistical analysis was carried out using the SPSS statistical software for Windows. The student *t*-test was used to determine the significant difference between the results. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Vesicles formation and CAT encapsulation efficiency (CAT EE)

Enzymes are very sensitive to their surrounding environment. As a method of immobilization, vesicle encapsulation presents an interesting solution because it provides a non-denaturing environment for enzyme protection [25]. In addition, encapsulation often offers mild conditions for enzyme biomolecule immobilization. Different SEs were chosen to evaluate the effect of fatty acid ester chain length and HLB values on the CAT EE. The chemical nature of the vesicles membranes may be manipulated to increase the drug loading by varying the additives used, which are typically Chol and/or PL. The results showed that in all tested samples, vesicles composed of SE:Chol:PL (10:1:10) had the lowest EE regardless of the type of SE used in the formulation of the vesicular dispersions. However, the vesicular dispersions of SE:Chol (1:1) had the highest CAT EE using 20 mg/ml SE concentration tested at three different vesicular compositions. The order of the EE results was S-1670 > P-1670 > M-1695 > L-1695 > S-970 > S-370 (Table 1). Addition of Chol to vesicles dispersions results in a better physical stability of the prepared vesicles [15]. The incorporation of Chol into the bilayer composition of the vesicles induces membrane-stabilization and decreases the leakiness of the membrane [26].

3.1.1. Effect of HLB

Regarding SEs, it is not sufficient to consider exclusively their fatty acids chain length, but the HLB values should also be taken into account. The results showed that along with the length of the fatty acid chains, the HLB value affects the CAT EE. SEs of lower HLBs had lower CAT EEs than SEs of higher HLBs (Table 1).

3.1.2. Effect of chain length

Fig. 1 shows the CAT EE of vesicles prepared with different SE/Chol-/PL ratios (10:1:10, 4:4:1, 1:1:0) related to the carbon-chain length of the SE fatty acid ester. It can be observed that the CAT EE increases with the increase in the carbon-chain length of the SE fatty acid ester from 12 to 18. These results could lead to the conclusion that when the encapsulated drug is as large as CAT, the longer alkyl chain was accommodated resulting in a high CAT EE. Similar findings were reported by Manosroi et al. [15], where the stearyl chain C18 non-ionic surfactant vesicles showed higher encapsulation efficiency than the lauryl chain C12. In addition, the membrane formed with stearyl chain surfactants was more stable even with low Chol concentrations than the membranes formed with surfactants of shorter alkyl chains [15].

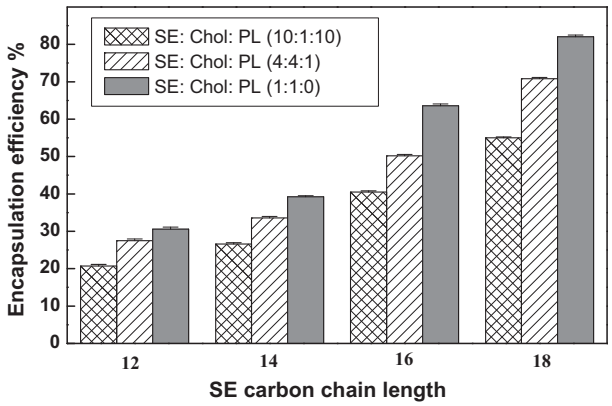

Fig. 1. The effect of SE carbon-chain length on the EE of CAT for different vesicles compositions of SE, Chol and PL. Vesicles were prepared using the thin-film hydration method at a fixed SE concentration (20 mg/ml). Each point represents the average of two experiments.

3.2. Influence of CAT initial concentration on EE

The relationship between CAT concentration and EE is linear, with EE increasing with increasing CAT concentrations up to 4 mg/ml. However, further increases in CAT concentration above 4 mg/ml did not produce a corresponding increase in encapsulated CAT. Recently, Alptekin et al. [14] reported similar observations upon catalase immobilization, where an initial amount of catalase above 8 mg/ml was reported to cause a decrease in the activity of the immobilized catalase.

3.3. Flexibility of SE-V

The flexible bilayer properties are governed by the system capability to sustain or respond to a (dynamic) mechanical stress. In most cases, it has been shown that classical vesicles are of little or no value as carriers for transdermal drug delivery; they do not penetrate the skin deeply, but rather remain confined to upper layers of the stratum corneum [27]. The values for the flexibility index for HSC, HSC–CAT, HSC–PL and HSC–PL–CAT after extrusion are reported in Table 2. The results indicated that the inclusion of PL increased the vesicles flexibility (1.3 times higher). PL has been previously reported to increase the flexibility of vesicles [28]. Considering PL- and Chol-containing vesicles, Chol prevents the crystallization of PL chains [29]. Therefore, PL in the presence of Chol is in a fluid state and retains chain flexibility [30]. The results showed that in both types of vesicles tested, the encapsulation of CAT did not have a significant effect on the vesicles flexibility ($p < 0.05$). HSC and HSC–CAT samples showed high flexibility-index values (3.46 ± 0.008 and 3.42 ± 0.01, respectively). This is consistent with the fact that when a surfactant is present in an adequate concentration, it can accommodate particle-shape deformation of the bilayer vesicles under stress.

Table 2
Flexibility index of sugar ester vesicles. Vesicles were prepared using the thin-film method at a final SE concentration 20 mg/ml. The flexibility index was determined by using the extrusion method.

Formulation	Vesicle composition SE/Chol/PL (w/w/w)	Flexibility index
HSC	1:1:0	3.46 ± 0.008
HSC–CAT	1:1:0	3.42 ± 0.01
HSC–PL	4:4:1	4.67 ± 0.2
HSC–PL–CAT	4:4:1	4.66 ± 0.1

Values are expressed as the mean ± SD ($n = 3$).

Table 3
The average particle size of empty sugar ester and CAT encapsulating vesicles and change in vesicle size after storage at 5 °C for 2 month as measured by laser diffraction. Vesicles were prepared using the thin-film method at a final SE concentration 20 mg/ml.

Formulation	Composition SE/Chol/PL (W/W/W)	Vesicle size (nm)	Size after 2 months of storage
HSC	1:1:0	184 ± 0.3	202 ± 0.32
HSC–CAT	1:1:0	222 ± 0.13	254 ± 0.24
HSC–PL	4:4:1	256 ± 0.29	264 ± 0.45
HSC–PL–CAT	4:4:1	275 ± 0.25	283 ± 0.29

Values are expressed as the mean ± SD ($n = 3$).

3.4. Verification of vesicle formation as examined by freeze fracture electron microscopy (FFEM)

All images of fractured SE-V vesicles (HSC and HSC–PL) and CAT-EVs (HSC–CAT and HSC–PL–CAT) exhibited a vesicular structure, which was confirmed by FFEM (Fig. 2). The morphological characterization of the SE-V by FFEM confirmed the formation of a spherical vesicular multilamellar structure. The images showed that CAT-EV did not alter or cause any deformation in the vesicles morphology. The SE-V and CAT-EV diameters were estimated with the smallest observed diameter of 170.8, 162.54, 167.4 and 166.7 nm for HSC, HSC–CAT, HSC–PL, and HSC–PL–CAT, respectively.

3.5. Vesicle size distribution

The mean sizes of the vesicle samples (HSC, HSC–CAT, HSC–PL and HSC–PL–CAT) as measured by LD, increased after 2 months of storage at 5 °C (Table 3). This size increment may be due to growth rather than aggregation because visual observation of the samples showed no aggregate, precipitation or phase separation upon storage at 5 °C. Similar observations were reported by Lawrence et al. [31] for niosomes using Brij and by Pardakhty et al. [32] for the formulation of niosomes for insulin delivery using Brij and Chol.

3.6. Catalytic kinetics

Table 4 shows the comparative data on the kinetic constants and catalytic efficiencies of both free and encapsulated CAT. The free CAT exhibited a catalytic efficiency value (V_{max}/K_m) of 0.07×10^4 mM H_2O_2/μmol H_2O_2 decomposed, while the CAT-EV exhibited a much higher catalytic efficiency of 0.12×10^4 mM H_2O_2/μmol H_2O_2 decomposed. The lower K_m value exhibited by CAT-EV indicated an increase in the enzyme affinity toward the substrate.

3.7. Thermal stability

Fig. 3A shows the thermal stability of free and encapsulated CAT examined after enzyme exposure to various temperatures ranging from 20 to 90 °C for 15 min prior to substrate addition. The CAT-EV showed high stability over the temperature range 20–50 °C with

Table 4
Kinetic parameters (K_m, V_{max}) and catalytic efficiency of free and encapsulated CAT.

Enzyme sample	K_m (mM)	V_{max} (μmol H_2O_2 decomposed/min) × 10^4	Catalytic efficiency (V_{max}/K_m) × 10^4
Free CAT	12.5	0.9	0.07
Encapsulated CAT	7.4	0.9	0.12

The 3.0 ml reaction mixture contained: 50 mM potassium phosphate buffer, pH 7.0, free or encapsulated CAT at a final concentration of 0.2 μg/ml protein and different concentrations of H_2O_2 ranging from 3 to 20 mM. Each point represents the average of two experiments.

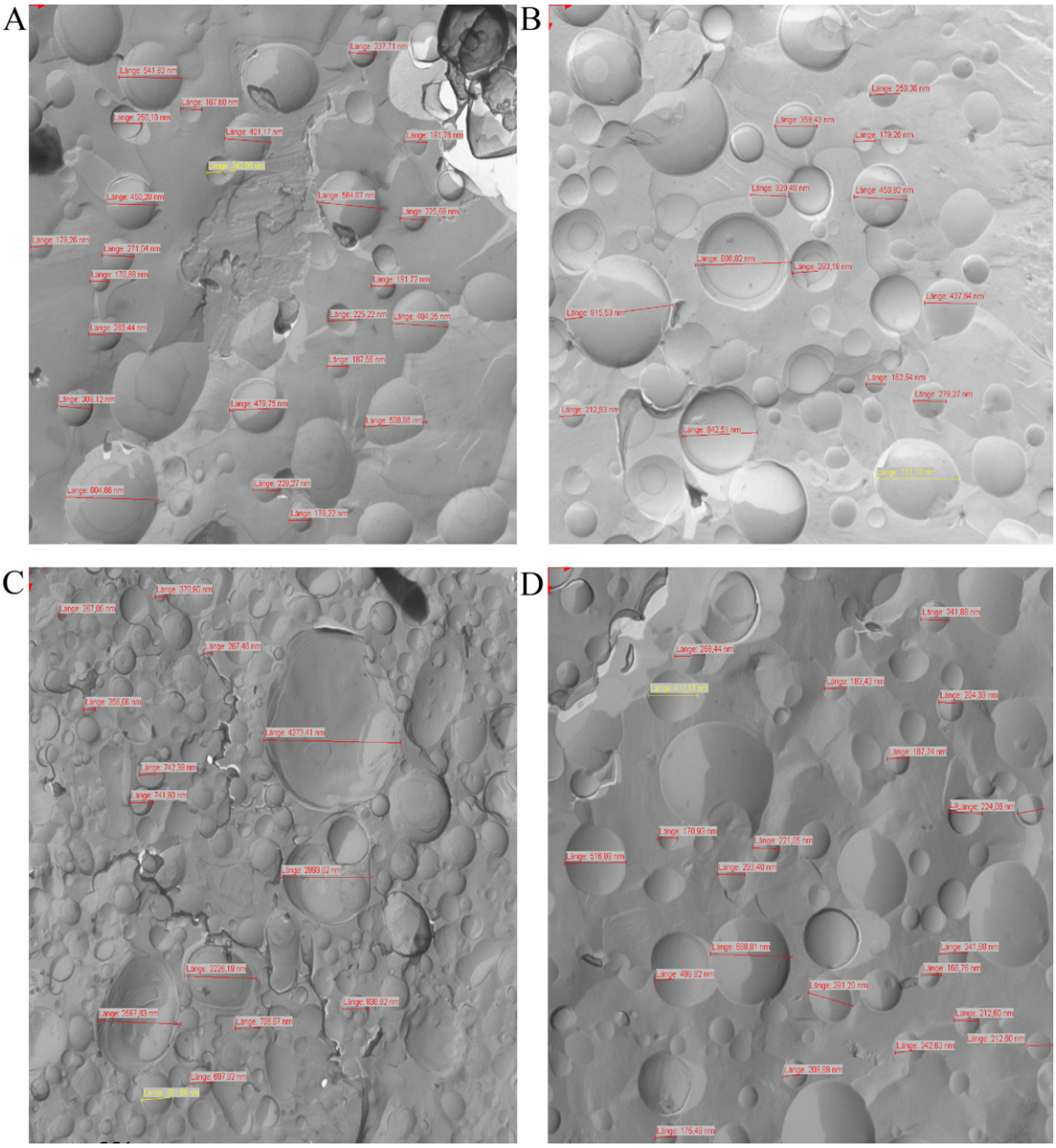

Fig. 2. FFEM images of SE-V and CAT-EV showing the vesicle diameters. (A) HSC (B) HSC–CAT (C) HSC–PL and (D) HSC–PL–CAT.

100% residual activity and 52% of activity was retained at 60 °C. The activity of the free CAT started to decline at 30 °C and reached 78.5% of its activity at 40 °C. The activity dropped dramatically when the working temperature was above 50 °C reaching 8% at 60 °C. Cetinus and Oztop [5] reported a 70% residual activity of immobilized catalase at 40 °C compared to 50% for the free form. In addition, catalase encapsulated in liposomes retained 83% of its initial activity after incubation at 55 °C, while free catalase showed significant deactivation [33].

3.8. pH optimum

Both free and encapsulated CAT showed maximum catalytic activity at pH 7.0 using potassium phosphate buffer. At all measured pHs, CAT-EV showed higher catalytic activity compared to the free form (Fig. 3B). These enhanced stability results can be attributed to the physico-chemical stability of the enzyme inside the SE vesicles. A similar conclusion was reached by Cetinus and Oztop [5], who reported that immobilized catalase activity was less sensitive to changes in pH than that observed for the free form.

3.9. Repeated decomposition of H_2O_2

3.9.1. Effect of vesicles composition on reusability

For immobilized enzymes, the reusability is considered an essential feature. Fig. 4A describes the relation between the residual activity % for the free and encapsulated CAT with HSC–P, HSC–PL and HSC, as a function of the number of successive reactions with repeated H_2O_2 addition. In all of the samples tested, there was a reduction in the catalytic activity with increasing numbers of repeated decomposition reactions. Data also show that the extent of the decrease in the catalytic activity of free CAT is higher than that for vesicular samples. Free CAT lost its catalytic activity completely after five successive decomposition reactions. However, after 12

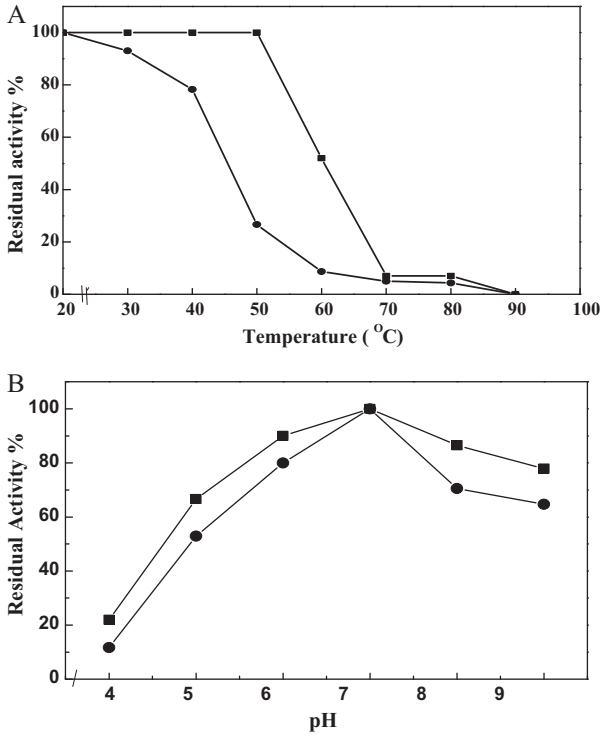

Fig. 3. The temperature stability (A) and pH optimum (B) of free (circle) and encapsulated CAT (square). For temperature stability, the enzyme activity was determined by measuring the residual activity after pre-incubation of the enzyme at the designated temperatures for 15 min. The enzyme activity was determined at various pHs [50 mM sodium acetate buffer (pH 4.0–5.0) and potassium phosphate buffer (pH 6.0–9.0)] to determine the optimum pH. Each point represents the average of two experiments.

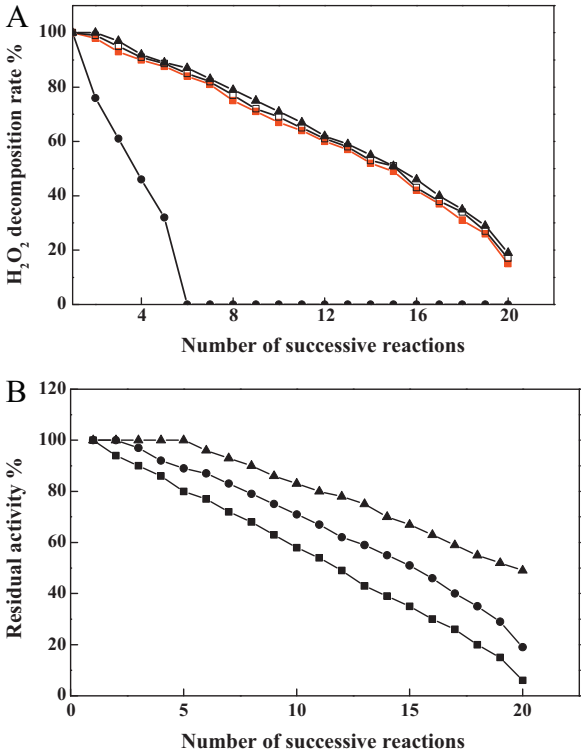

Fig. 4. (A) Effect of vesicle composition on reusability of catalase for successive decomposition of H_2O_2. The samples used included free CAT (circle), HSC–P (closed square), HSC–PL (open square) and HSC (closed triangle). (B) Effect of the CAT-EV concentration on reusability for successive decomposition of H_2O_2. CAT concentrations were 0.02 μg/ml (closed square), 0.2 μg/ml (circle) and 50 μg/ml (closed triangle). Aliquots of a freshly prepared 0.15 M H_2O_2 solution were added to reproduce the initial reaction conditions in each successive catalytic reaction. Each run of successive decomposition reactions was performed in duplicate.

decomposition cycles, the comparable residual activities for CAT with HSC–P, HSC–PL and HSC were found to be 60, 61 and 62%, respectively. Therefore, the concentrations of SE, Chol and PL used for vesicles preparation showed no effect on CAT reusability. Similarly, immobilized catalase was used for 10 successive cycles in the destruction of 10 mM H_2O_2 without a significant decrease in enzyme activity [34].

3.9.2. Effect of CAT-EV concentration on reusability

The effect of CAT concentrations on its reusability in the repeated decomposition of H_2O_2 was studied (Fig. 4B). The results obtained showed that the residual activity in CAT-EV (CAT–HSC) increased with increasing concentration of CAT. CAT-EV at 50 μg/ml showed a higher reaction stability in the repeated H_2O_2 decomposition than the lower concentrations tested (0.02 and 0.2 μg/ml).

3.10. Effect of trypsin

Catalase enzyme has been reported to lose its catalytic activity upon digestion by trypsin enzyme [35]. Fig. 5 shows the residual catalytic activity of CAT as a function of increasing trypsin concentrations. In the case of free CAT, the addition of 0.5 mg trypsin/ml caused an 80% loss in catalytic activity. CAT-EV, on the contrary, showed resistance against the proteolytic effect of trypsin, and no reduction in catalytic activity was detected upon addition of 0.5 mg/ml trypsin. At higher trypsin concentration (4 mg/ml), a 20% catalytic activity was retained for CAT-EV.

3.11. Storage stability of free and encapsulated CAT

3.11.1. Evaluation of physical stability

The physical stability of CAT-EV was evaluated at 5 °C and monitored over a period of 2 months. A visual inspection showed no precipitations, phase separation or aggregation of the vesicular dispersion.

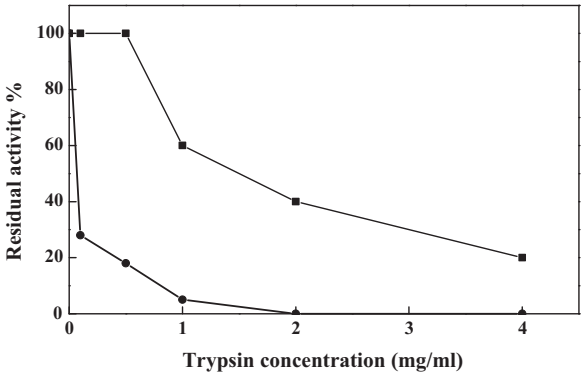

Fig. 5. The effect of trypsin on the catalytic activity of free (circle) and encapsulated CAT (square). The residual activity was determined after pre-incubation of free or encapsulated CAT for 2 h with different trypsin concentrations (0–4 mg/ml). The catalytic activity was measured under standard assay conditions. Each point represents the average of two experiments.

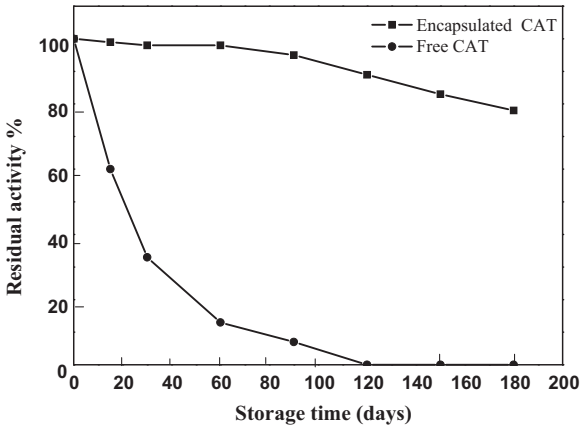

Fig. 6. The storage stability of free and encapsulated CAT. The enzymes were stored at 5 °C in 50 mM potassium phosphate buffer, pH 7 at a final CAT concentration of 4 mg/ml. The catalytic activity was measured under standard assay conditions. Each point represents the average of two experiments.

3.11.2. Evaluation of catalytic stability

The shelf life of an enzyme-based anti-oxidant preparation depends on how long the biological activity of the enzyme can be retained, and this time may vary from days to months. Free CAT and CAT-EV at 4 mg/ml final concentration were stored in 50 mM potassium phosphate buffer pH 7, over a period of 6 months and the catalytic activity was measured at different time intervals at 5 °C (Fig. 6). CAT showed a much higher stability results upon encapsulation, where it was stable for a period of 90 days with 95% residual activity. However, free CAT retained 7% of its activity after 90 days. These experimental results indicated that encapsulation stabilizes the enzyme in comparison to the free counterpart. These results are in agreement with previous studies, which reported enhanced storage stability for catalase upon encapsulation in liposomes [33] and immobilization on chitosan film [5] and Eupergit C [14].

3.12. Model drug efficiency in wound healing

Although many studies in the medical sciences reported enhanced wound healing using systemic free radical scavengers, topical application was reported only by a few authors [4]. In this study, the thermal injury model was chosen to study the effect of topical CAT application in diminishing the oxidative injury to the skin. At Day 0 after burn wounding, all wounds from all groups exhibited a statistically identical degree of inflammation. After 24 h, the treated group (CAT-EV) showed a significant ($p < 0.05$) decrease in lesion size compared to the naive (untreated) and control groups (SE-V). Comparing the lesion sizes to the initial size (100%), the lesion size of the naive and control groups extended to 135% and 120%, respectively (Table 5). The lesion sizes in the treated groups did not show a significant increase in size from the initial size. Highly significant differences in wound healing were observed visually after 48 h for the treated groups. In addition, the lesion size reduction and rate of wound healing progression was much higher in the treated group after 72 h. The data obtained show that topical treatment using antioxidant CAT-EV as a defense mechanism may offer a good strategy for the treatment and prevention of aging and photoaging in human skin. The decreased activity of CAT in the dermis of photoaged and aged human skin *in vivo*, are believed to cause an age-associated increase in ROS in aged skin [6]. Consequently, ROS, such as hydrogen peroxide, may increase and accumulate in aged skin; these ROS will affect the signaling pathways and eventually lead to aged and photoaged skin *in vivo* [36]. Giacomoni and Rein [37] established that one might have better success in improving skin repair and fighting skin aging by developing treatments that maintain the redox balance in the skin layers.

Table 5
The effect of CAT-EV on wound healing *in vivo*.

Group	Number	Treatment administrated	Size of lesions (cm^2, after 24 h)
Nave	6	–	8.41 ± 0.10
Control	6	Empty vesicles	7.25 ± 0.13
Treated	6	CAT vesicles	6.25 ± 0.07

The thermal injury model was used for trauma induction. The three groups of male Wistar rats consisted of six animals each. Treatment with CAT-EV was performed immediately after trauma and once daily for 4 days (treated group). The average CAT content in CAT-EV applied was 4 mg/ml. One group of animals was left untreated (naive group) and one was treated using empty SE-V (control group).

4. Conclusions

The notion that a targeted antioxidant prophylaxis may be created using immobilized CAT is extremely important. This innovative work demonstrated the feasibility of the preparation of flexible biocompatible nano sugar ester vesicles. The encapsulation of CAT in the vesicles enhanced its kinetic parameters, pH, thermal stability, reusability and protection against trypsin. New opportunities for the topical application of enzymatic radical scavengers CAT-EV in post-burn damage were confirmed *in vivo*. In conclusion, the flexible nano sugar ester vesicles developed in this study are a novel addition to the clinical and industrial arsenal for safe and active CAT topical photoprotective applications.

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